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Antisera to selected outer membrane proteins of *Vibrio cholerae* protect against challenge with homologous and heterologous strains of *V. cholerae*

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Abstract

Each year cholera epidemics occur in various places around the world. Though there is no effective vaccine against cholera, people who recover from an infection usually have prolonged immunity to the disease. Sera from convalescent patients contain antibodies to a number of outer membrane proteins (OMPs) of *V. cholerae*. We isolated several OMPs (43, 42, 30, and 22 kDa) from *V. cholerae* V86 El Tor Inaba, sequenced their amino-termini, and generated hyperimmune sera against them in rabbits. Antisera to the 43-, 42-, and 22-kDa OMPs, but not the preimmune sera, significantly reduced *V. cholerae*-induced fluid secretion seen in rabbit intestinal loops challenged with the homologous strain. In addition, a combination of antisera to the different OMPs reduced the fluid secretion induced by challenge with heterologous *V. cholerae* Ogawa and O139 strains. These results have significance in the development of vaccines to *V. cholerae*, as the hyperexpression of these OMP encoding genes in vaccine strains may improve the efficacy of cholera vaccines.

Keywords: *Vibrio cholerae*; Immunogenic surface protein; Cholera vaccine

1. Introduction

Every year, cholera epidemics are seen in parts of the world where the disease is endemic and in regions where the sanitation facilities have broken down due to civil unrest or economic upheaval. According to the World Health Organization (WHO), in 1996 there were 143 349 cases of cholera worldwide that resulted in 6689 deaths, mostly in children [1]. Patients who recover from cholera usually have a prolonged immunity against the infecting serotype and to heterologous serotypes.

During an infection, patients develop antisera to both lipopolysaccharide (LPS) and proteins on the bacterial surface. It has been shown that the protein antigens provide the prolonged immunity against homologous and heterologous serotypes of *Vibrio cholerae* [2]. The early vaccines against cholera contained *V. cholerae* cells killed with formalin, which is detrimental to the protein antigens. As a result, the immunity invoked by vaccinees was predominantly

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against the LPS, and it was short-lived [3]. In recent years, two live, attenuated vaccine strains of *V. cholerae* have been tested extensively in cholera endemic and non-endemic areas [4,5]. Vaccine strain CVD 103-HgR was derived from *V. cholerae* 569B Classical Inaba by deleting the gene for the A subunit of cholera toxin (*ctxA*) and introduction of a gene encoding resistance to mercury (Hg^{2+}) [4]. Vaccine strain CVD 110 was constructed from *V. cholerae* E7946 El Tor Ogawa by deleting the toxin genes *ctxA*, *zot* (zona occludens toxin), and *ace* (accessory colonizing enterotoxin) [5]. CVD 103-HgR has been successful against homologous and heterologous challenge with the classical biotype of *V. cholerae* in both children and adults [6], and was 60% protective efficacy against *V. cholerae* El Tor [4]. When CVD 110 was tested in healthy adult volunteers, though it was highly immunogenic, it produced diarrhea and other adverse reactions [7]. Since the predominant *V. cholerae* biotype of the current pandemic is El Tor, it is necessary to develop vaccines that provide better immunity against this strain. Moreover, CVD 103-HgR does not protect against *V. cholerae* O139, the new strain of *V. cholerae* that emerged in 1992 [8].

Therefore, we opted to identify specific outer membrane proteins (OMPs) that are immunogenic and could provide protection against cholera. After studying the OMP profile of *V. cholerae* V86 (El Tor, Inaba), we selected four major OMPs that were 43, 42, 30 and 22 kDa in size and developed antisera against them in rabbits. We used the rabbit ligated ileal loop model to evaluate the protective efficacy of the antisera. The rabbit ileal loop model, developed in 1959, is an accepted animal model that mimics the intestinal hypersecretion characteristic of clinical cholera [9]. The fluid accumulating in the intestinal loops is an indication of the virulence of the *V. cholerae* and the susceptibility of the host against the infection. In this study, we challenged rabbit intestinal loops with live *V. cholerae* cells that had been preincubated with the antisera or with preimmune serum. We used both the homologous strain, *V. cholerae* V86, and two heterologous strains, *V. cholerae* Ogawa and *V. cholerae* O139. In all cases, the antisera against OMPs of 43, 42, and 22 kDa reduced fluid secretion in rabbit intestinal loops challenged with live *V. cholerae* cells.

2. Materials and methods

2.1. Bacterial strains

V. cholerae V86 El Tor Inaba is a clinical isolate [10] that has been maintained in the laboratory at −70°C. *V. cholerae* 3008 El Tor Ogawa was obtained from Dr. J.P. Nataro at the Center for Vaccine Development, Baltimore, MD. The *V. cholerae* O139 strain was obtained from the International Centre for Diarrhoeal Disease Research in Dacca, Bangladesh.

2.2. Isolation of OMPs, SDS-PAGE, and protein sequencing

The OMPs of *V. cholerae* were isolated following the procedure described by Filip et al. [11]. The OMPs were separated on a 12% polyacrylamide gel run in the presence of 2-mercaptoethanol and stained with Coomassie blue [12]. For amino-terminal sequencing, the proteins were electroblotted onto an Immobilon-P membrane (Millipore, Houston, TX) which was stained with Coomassie blue. The amino-termini of the stained bands were sequenced at the Protein Chemistry Core Laboratory (UTMB) using an Applied Biosystems 475A amino acid sequencer.

2.3. Generating antisera to selected OMPs

The OMPs were separated on 12% SDS-polyacrylamide gels and stained with Coomassie blue. The bands representing the desired proteins were excised, chopped into small pieces, mixed with Freund's complete adjuvant, and injected subcutaneously into rabbits. Blood samples were drawn from the rabbits before the first immunizing dose and used as the preimmune serum. Subsequent immunizations were performed with protein mixed in Freund's incomplete adjuvant every 4–5 weeks after the first immunization. Blood was drawn from the rabbits prior to boosting on days 31, 79, 101, and 143 and designated B1, B2, B3 and B4. Titers of the anti-OMP sera were determined by enzyme-linked immunosorbent assay (ELISA) using the *V. cholerae* V86 OMP preparation as antigen (4 μg per well) and goat anti-rabbit IgG conjugated to alkaline phosphatase as the second

antibody (Bio-Rad, Hercules, CA). The reaction was developed with substrate (*p*-nitrophenyl phosphate, disodium, 1 mg ml^{-1}; Sigma, St. Louis, MO) and measured in an ELISA plate reader (Molecular Devices, Menlo Park, CA) at OD_{405}.

2.4. Rabbit ileal loop challenge

V. cholerae cells growing in exponential phase were centrifuged (4000×*g*, 10 min) and resuspended in peptone saline. A Klett-Sumnerson colorimeter was used to adjust the concentration of cells to 2×10^5 cfu ml^{-1} (a Klett reading of 170 represents 2×10^9 cfu ml^{-1}). The *V. cholerae* cells (1×10^5 cfu/loop) were incubated with 0.5 ml of preimmune or immune sera at 37°C for 1 h and then loaded into syringes to be injected into the rabbit intestinal loops. The complement in the antisera was inactivated by incubation of the sera at 56°C for 30 min before being added to the *V. cholerae* cells.

Female, New Zealand White rabbits, weighing 1.5–2 kg, were obtained from Myrtle's Rabbitry Inc. (Thompson Station, TN). The rabbits were housed and maintained using guidelines established by the Animal Care and Use Committee (ACUC) at the University of Texas Medical Branch in Galveston, TX. The rabbits were placed on a liquid diet of Sustacal® for 3 days and then fasted overnight the day before the intended surgery. The rabbits were anesthetized (35 mg kg^{-1} ketamine HCl and 5 mg kg^{-1} xylazine), and the small intestine was exposed through a midline incision in the abdomen. The intestine was lavaged with warm phosphate buffered saline (PBS) and ligated into loops of approximately 8 cm. Each loop was then injected with either *V. cholerae* cells, *V. cholerae* cells that had been preincubated with heat-inactivated sera, or with peptone-saline (0.1% peptone in 0.85% NaCl). The intestines were returned to the abdominal cavity and the incision was sewn shut. The rabbits were housed for 16–18 h (overnight) and then killed by rapidly injecting Nembutal (1 cm^3) into the marginal ear vein. The intestinal loops were removed from the rabbits, and the length of each loop and the quantity of fluid measured. The fluid accumulation was recorded as ml cm^{-1} and the protection afforded by the antisera expressed as percentage of fluid reduction compared to that of the preimmune serum.

2.5. Data analysis

The fluid accumulation results were evaluated with Student's *t*-test, using paired samples to determine whether protection was afforded by the immune sera compared to preimmune sera.

3. Results and discussion

3.1. The OMP profile of V. cholerae

The OMPs of *V. cholerae* were isolated, separated on a 12% SDS-polyacrylamide gel, and stained with Coomassie blue (Fig. 1). Many of the proteins reacted with convalescent sera from cholera patients in Western blots (data not shown). Based on the SDS-PAGE and the Western blot data, we selected four OMPs (43, 42, 30 and 22 kDa) that reacted with the sera from convalescent cholera patients and were consistently present in all preparations in reasonable amounts.

When the amino-terminal sequences of these proteins were determined (Table 1) and compared with sequences that were available in SwissProt and GenBank, we found homology with previously identified *V. cholerae* surface proteins. The amino-terminal sequence of the 22 kDa OMP was identical to the deduced amino acid sequence of the *ompW* gene that had been cloned and sequenced earlier [13]. However, the role of this gene product in the patho-

Table 1
Amino-terminal sequences of the selected OMPs from *V. cholerae* V86

Molecular size (kDa)	Amino-terminal sequence	Homology to *V. cholerae* proteins	Function
43	A E I L K S D A G T V D F Y G Q L R T	No known homology	unknown
38–42	D G I N Q S G D K A G S T V Y D A K G	OMPU	porin/adhesion protein
30	E V Y V G G K V G W S D L D D A X L A	OMPA	unknown
22	H Q E G D F I V R A G I A S V V P	OMPW	unknown

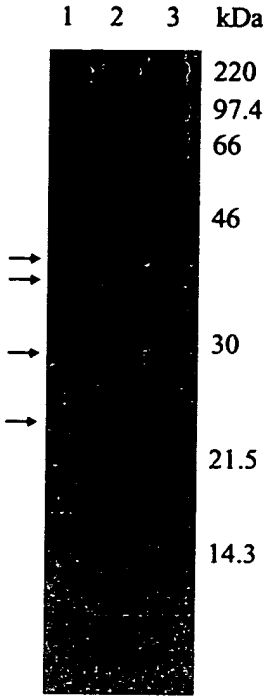

Fig. 1. Outer membrane protein profile of *V. cholerae* V86. This is a 12% SDS-polyacrylamide gel of the OMPs of *V. cholerae* stained with Coomassie blue. Arrows indicate the OMPs chosen for this study (43, 42, 30 and 22 kDa). Lanes 1 and 2: OMPs; lane 3: Molecular mass markers.

genesis of *V. cholerae* infections has not been elucidated. The 42- and 30-kDa OMPs recently were identified as OMPU and OMPA, respectively ([14] and GenBank accession number U73751). OMPU was thought to be an adhesion protein in the porin family [15]. The active form of OMPU consisted of three identical subunits. OMPU formed a pore of 1.6 nm with an exclusion limit of about 850 Da [15]. The function of OMPA is not known at this time. There was no homolog for the 43-kDa OMP.

3.2. Immunogenicity of the selected OMPs

In order to determine whether the selected OMPs (43, 42, 30 and 22 kDa) would be immunogenic, we generated polyclonal antibodies in rabbits to each of the OMPs. The rabbits developed significant antibodies to the OMPs as determined by ELISA (data not shown). However, the titer of antibodies to the 30-kDa OMP was relatively low compared to the other OMPs.

3.3. Protection against homologous V. cholerae challenge

After antibodies were generated to denatured polypeptides, we examined the capacity of these antisera to provide protection against fluid secretion in rabbit ligated ileal loops challenged with *V. cholerae*. The results, summarized in Table 2, indicate that antisera against the 43-, 42-, and 22-kDa OMP significantly reduced ($P < 0.05$) the fluid secreted into the rabbit intestinal loops as a result of infection by live *V. cholerae* cells. The mean reduction in fluid secretion seen in five rabbits was 90, 84 and 72% respectively. In contrast, antisera to the 30-kDa OMP did not protect against fluid secretion. The ligated loops challenged with *V. cholerae* cells incubated with preimmune serum showed no protection and caused fluid secretion identical to that seen with loops challenged with *V. cholerae* cells in peptone-saline.

Table 2
Antisera to selected OMPs protect rabbit intestinal loops challenged with live *V. cholerae* V86 cells

Challenge organism and sera	Fluid accumulation (ml cm^{-1}) ± S.D.		Protection (%)
	Preimmune	Immune	
V. cholerae and antisera to the 43-kDa OMP	0.75 ± 0.21	0.08 ± 0.07	86.60*
V. cholerae and antisera to the 42-kDa OMP	0.82 ± 0.30	0.15 ± 0.15	82.15*
V. cholerae and antisera to the 30-kDa OMP	0.81 ± 0.26	0.82 ± 0.13	–
V. cholerae and antisera to the 22-kDa OMP	0.91 ± 0.22	0.24 ± 0.22	57.14*
V. cholerae cells, no antisera	1.2 ± 0.53		
Peptone-saline, no bacteria or antisera	0.06 ± 0.1		

Protection = [(fluid accumulation with preimmune sera − fluid accumulation with immune sera)/fluid accumulation by preimmune sera] × 100. The data were analyzed by paired *t*-test. *$P < 0.01$. The data in each cell are the average from five different animals.

In the next set of experiments, we tested the antisera against the 43-, 42-, and 22-kDa OMPs. The methods used in these experiments were similar to those of the first set of experiments, with the exception that 1.5 ml of 2×10^5 cfu of *V. cholerae* cells was mixed with 0.5 ml each of the antisera to the 43-, 42-, and 22-kDa OMPs. This experimental design was to ensure that the ratio of cells to sera would remain the same as in the previous experiment. The data obtained from *V. cholerae*-challenged rabbits are summarized in Table 3. There was a significant reduction ($P<0.05$) in fluid accumulation. Four of the nine rabbits showed total protection, with no fluid in the loops.

In order to confirm the protection afforded by the hyperimmune sera to the OMPs, we diluted the mixed antisera 1:10, 1:50 and 1:100 in PBS before mixing it with the *V. cholerae* cells. A significant reduction ($P<0.05$) in fluid secretion was observed, compared to those loops challenged with *V. cholerae* cells that had been incubated with preimmune serum (Table 3). There was a decline in the protective efficacy of the antisera with dilution, and the reduction in fluid secretion was not statistically significant after the 1:100 dilution (data not shown).

3.4. Protection against heterologous challenge

Published reports about convalescent cholera patients have asserted that protein antigens provide prolonged protection against homologous and heterologous strains of *V. cholerae* [3,16]. In the experiments described thus far, *V. cholerae* V86 Inaba was used as the challenge agent. This was the same serotype and strain of *V. cholerae* from which the OMPs had been extracted. We next tested pooled antisera to selected OMPs for protection against challenge with heterologous strains of *V. cholerae*. Since *V. cholerae* V86 was of the Inaba serotype, we used an Ogawa serotype (*V. cholerae* 3008 Ogawa) for challenge experiments. We also used *V. cholerae* O139, the new epidemic strain of *V. cholerae* [17]. The protective efficacy of these antisera against the *V. cholerae* Ogawa and O139 was significant ($P<0.05$) (Table 3). The reduction in fluid secretion seen against the Ogawa serotype (73%) was comparable to that seen against V86 (87%). The protective efficacy against *V. cholerae* O139, though statistically significant, was not very impressive at 18%. Therefore, these OMPs would not be the best choice to develop protective immunity against *V. cholerae* O139. *V. cholerae* O139 has an OMP profile similar to that of *V. cholerae* El Tor, but it also has a capsule around the bacterial cell that may restrict access of the antibodies to the OMPs and interfere with their ability to bind to the OMPs [17,18].

In conclusion, we have shown that antisera to the 43-, 42-, and 22-kDa OMPs but not to the 30-kDa OMP of *V. cholerae* protected to varying degrees against intestinal challenge with live *V. cholerae* cells of both homologous and heterologous serotypes. Since El Tor is the most common biotype in the cholera outbreaks today, it is important to develop strategies that increase the efficacy of vaccines

Table 3
Protection of rabbit intestinal loops against challenge with homologous and heterologous *V. cholerae* strains by antisera to the selected OMPs

Organism	Serum	Fluid accumulation (ml cm^{-1}) ± S.D.	Protection (%)
V. cholerae V86 El Tor Inaba	Preimmune[a]	1.30 ± 0.52	
	Immune[a]	0.24 ± 0.34	87.00*
	Immune serum diluted 1:10[a]	0.26 ± 0.36	83.64*
	Immune serum diluted 1:50[a]	0.64 ± 0.31	51.73*
	Immune serum diluted 1:100[a]	0.93 ± 0.40	28.65§
V. cholerae O139	Preimmune[b]	1.13 ± 0.50	
	Immune[b]	0.92 ± 0.42	18.2§
V. cholerae 3008 El Tor Ogawa	Preimmune[b]	1.76 ± 0.31	
	Immune[b]	0.46 ± 0.20	73.38*

The immune serum was a mixture of antisera to the 43-, 42-, and 22-kDa OMPs. The preimmune serum was a mixture of the sera obtained before immunization. The sera were mixed 1:1:1. *$P<0.001$: §$P<0.01$: a: $n=9$; b: $n=5$. Protection = [(fluid accumulation with preimmune sera − fluid accumulation with immune sera)/fluid accumulation with preimmune sera] × 100.

against this biotype. The results of our experiments showed that the OMPs we tested were highly immunogenic and protective. Genes encoding these proteins could be expressed in an oral bacterial vaccine strain such as *Salmonella typhi* Ty21A, to produce a multivalent vaccine. Moreover, since these are protein antigens, they will trigger immunological memory and provide long-lasting protection, as seen after infection with *V. cholerae*. The protection against *V. cholerae* in these experiments was provided by serum IgG antibodies and showed that raising antisera against OMPs was protective. Mucosal immunity plays an important role in the protection against cholera, therefore these data provide a basis for future studies to evaluate the role of these OMPs in the mucosal response to *V. cholerae*.

There are so many diseases that children have to be immunized against that vaccine developers are always looking for ways to combine the vaccinations. Since these OMPs are immunogenic, they could be engineered with neutralizing epitopes from other bacteria that would then be expressed on the surface of *V. cholerae*. This strategy would aid in the development of a multi-component vaccine effective against various infectious diseases.

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